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DNA-binding protein(s) interacts with a conserved nonameric sequence in the upstream regions of wheat histone genes

Takefumi Kawata, Takuya Nakayama, Koji Mikami, Tetsuya Tabata, Hisabumi Takase and Masaki Iwabuchi

Department of Biology, Faculty of Science, Hokkaido University, Sapporo 060, Japan

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A nuclear protein(s), HBP-2, that binds to the upstream region of the wheat histone H4 gene was identified from a fractionated nuclear extract of wheat germ by DNase I footprinting. The DNase I-protected region contained the conserved nonameric motif, CATCCAACG. Cross-competition experiments that used the mobility shift assay showed that this nuclear protein(s) binds specifically to the upstream sequence that has been postulated to be a *cis* element of the wheat H3 gene. Our findings suggest that this DNA-binding protein(s) may be a *trans*-acting factor in the regulation of the transcription of wheat histone genes.

Histone gene; Nonameric motif; DNA-binding protein; Mobility shift assay; DNase I footprint; (Wheat)

1. INTRODUCTION

Recent studies of the transcriptional regulation of animal and viral genes have demonstrated that *cis*-acting regulatory elements control the transcription level of genes through cooperative action with *trans*-acting protein factors [1,2]. Much less information about the *trans*-acting factors of plant genes, however, is available than for animal and viral genes.

In studying the regulatory mechanism for the transcription of wheat histone genes, we found the candidates for *cis*-acting elements and *trans*-acting protein factors that specifically interact with the presumed *cis*-elements of the histone genes ([3–7]; Nakayama, T. et al., unpublished; Kawata, T. et al., unpublished; Mikami, K. et al., unpublished). We previously reported a DNA-binding protein(s) HBP-1 that specifically interacts with the hexameric sequence that serves as a *cis*-element in the wheat H3 and H4 genes [7]. We have now demonstrated the existence of another DNA-binding protein(s), HBP-2, which specifically interacts with a definite sequence located between the TATA box and the HBP-1 binding site. This protein-binding region agrees with one of the *cis*-elements found in the wheat H3 gene (Nakayama et al., unpublished) and contains the conserved nonameric sequence CATCCAACG that is present in certain other plant genes [8–13]. We concluded that the nonameric motif and its DNA-binding protein(s) must act as regulatory elements in the transcription of some plant genes.

Correspondence address: M. Iwabuchi, Department of Biology, Faculty of Science, Hokkaido University, Sapporo 060, Japan

2. MATERIALS AND METHODS

2.1. *Preparation of nuclear extracts and chromatographic purification of nuclear proteins*

A crude nuclear extract was prepared from 8 g (dry wt) wheat germ, as described [7]. The scheme for the partial purification of nuclear proteins is shown in fig.1A. The extract (2 ml), adjusted to 20% (w/v) $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$, was stirred and then centrifuged. The supernatant obtained was adjusted to 60% (w/v) $(NH_4)_2SO_4$. The precipitates were collected by centrifugation and suspended in buffer A [20 mM Hepes-KOH, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM DTT] containing 0.1 M KCl then dialyzed against solution A (buffer A plus 0.1 M KCl). The dialyzate was applied to a

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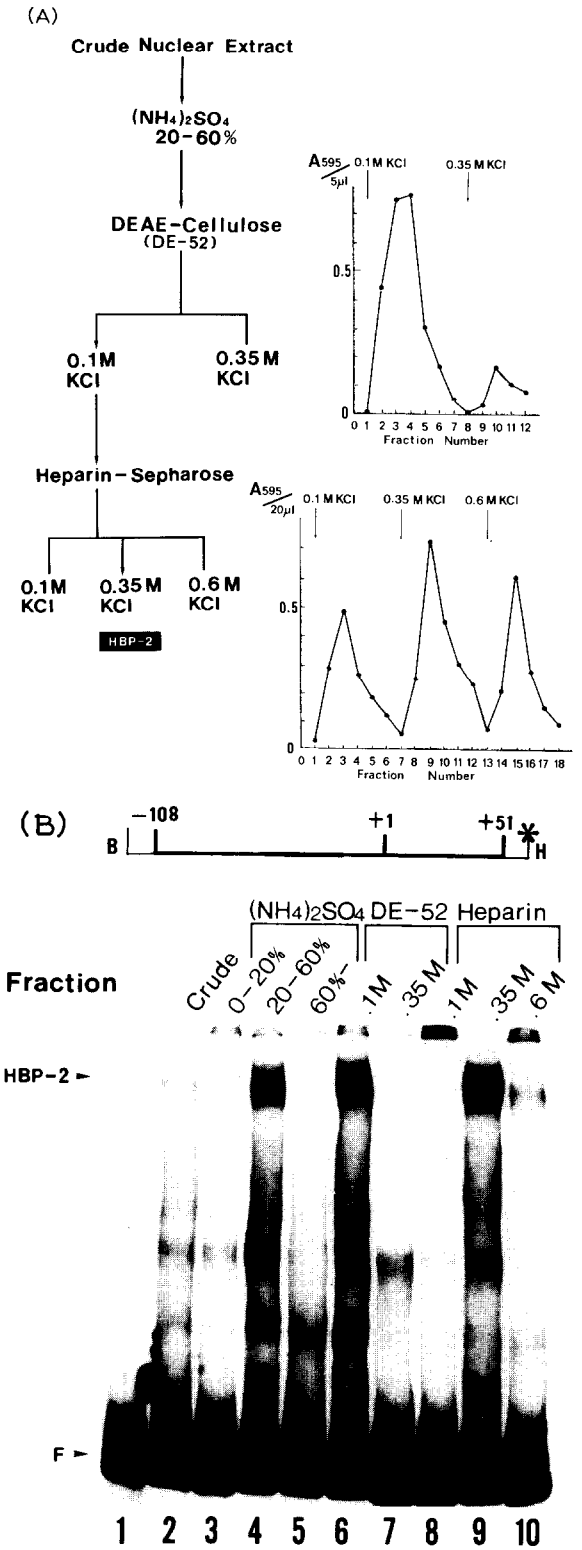

Fig.1. (A) Scheme for the partial purification of DNA-binding protein(s) from nuclear extracts of wheat germ. Elution profiles of the extract obtained by chromatography on DEAE-cellulose (upper) and heparin-Sepharose (lower) columns are shown on the right. A_{595}: absorbance of proteins at 595 nm as determined by the Bradford method. (B) DNA-binding activities of fractionated extracts detected by the mobility shift assay. The H4 probe (−108 to +51) is indicated at the top: bold line, part of the H4 DNA; thin line, M13mp19 polylinker; B, *Bam*HI; H, *Hin*dIII; *, the ^{32}P label. Fractions used in the mobility shift assay are indicated at the tops of the lanes. Lanes: 1, free probe; 2–10, DNA-binding assays in the presence of each fraction (3 μg protein/assay). F, free probe; HBP-2, specific DNA-protein(s) complex.

DEAE-cellulose (Whatman DE-52) column (0.8 × 5 cm) equilibrated with solution A, and the loaded column, washed with the same solution. Proteins were eluted stepwise from the column with two column volumes each of 0.35 and 0.6 M KCl in buffer A. Fractions (0.5 ml) of the protein peak were pooled and dialyzed against buffer A. The flow-through fraction from the DE-52 column was applied directly to a heparin-Sepharose (Pharmacia) column (0.8 × 2.5 cm) equilibrated with solution A, and fractions eluted stepwise with 0.1, 0.35, 0.6 and 1.0 M KCl in buffer A. Fractions (400 μl) that contained the protein peaks were pooled and dialyzed against buffer B (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.02% Nonidet P-40. Proteins were quantified according to the method of Bradford [14].

2.2. *Probes and competitors for mobility shift assays and DNase I footprint assays*

To prepare the H4 probe (−108 to +51), we digested pM13H4 (−108/+51)RF with *Hin*dIII and labeled its unique *Hin*dIII site with [α-^{32}P]dCTP using the Klenow fragment for the top strand and with [γ-^{32}P]ATP using T4 polynucleotide kinase for the bottom strand. Labeled RF-DNA was digested with *Bam*HI, and the fragment (−108 to +51) isolated. A specific H4 competitor was prepared from pM13H4(−108/+51)RF by double-digestion with *Hin*dIII and *Bam*HI. As the specific H3 competitor, an *Eco*RI/*Hin*dIII fragment [−167 to −41 in the H3 gene (pTH012)], which contains an M13 polylinker, was obtained from pM13H3(3′Δ-41) (unpublished). The polylinker fragment from pUC118 and the 20 nucleotide oligomers that contain the hexameric and octameric motifs of the wheat H3 gene, d(5′-TCGGCCACGTCACCAATCCG-3′) and d(5′-CCAATCCGCGGCATTCCATC-3′), were used as nonspecific competitors.

2.3. *Mobility shift and DNase I footprint assays*

The mobility shift assay was performed with an H4 probe as described ([7]; Kawata, T. et al., unpublished]. For the DNase I footprint assay, ^{32}P-labeled probes (4 fmol) were incubated for 20 min at 25°C with the 0.35 M KCl heparin-Sepharose fraction in a total volume of 20 μl that contained 17 mM Hepes-KOH, pH 7.9, 60 mM KCl, 7.5 mM $MgCl_2$, 0.12 mM EDTA, 17% glycerol, 0.6 mM PMSF, 1.2 mM DTT and 0.5 μg of poly(dI-dC)·poly(dI-dC). After incubation, 1 μl of DNase I (150–200 μg/ml) was added and digestion allowed to proceed

for 30 or 60 s at 25°C. The DNase I was inactivated by the addition of 5 μl of a stop solution (1.5 M Na acetate, pH 5.2, 20 mM EDTA and 100 μg/ml sonicated calf thymus DNA). DNA fragments were immediately extracted with phenol, after which they were precipitated with ethanol then electrophoresed on denaturing sequence gels.

3. RESULTS AND DISCUSSION

To detect protein(s) that bind(s) to the upstream region of wheat histone H4 gene, we performed mobility shift assays of chromatographically fractionated nuclear extract with the H4 probe (−108 to +51). A major protein(s)-DNA complex was found in the 20–60% ammonium sulfate precipitate, 0.1 M KCl DE-52, and 0.35 M KCl heparin-Sepharose fractions (fig.1B). Formation of this complex was specific for the H4 probe because the addition of specific H4 competitor prevented formation (fig.3, lane 3). We have designated the protein(s) involved in the formation of this complex HBP-2 (histone-DNA-binding protein(s)-2). We could not determine the degree of purification of HBP-2 as we lacked adequate criteria with which to measure its DNA-binding activity. The HBP-2 content was enriched, however, as purification proceeded because formation of other nonspecific complexes gradually decreased (cf. lanes 2, 4, 6 and 9 in fig.1B). The DNA-binding activity of HBP-2 was recovered in the 0.35 M KCl fraction after heparin-Sepharose column chromatography (fig.1).

To determine the HBP-2-binding site(s) on the H4 probe, we carried out a DNase I footprinting assay. The DNase I-protected area on the bottom strand was located between positions −82 and −60 and that on the top strand between positions −78 and −58 (fig.2). Interestingly, the sequence in the protected area is also present in the corresponding region of the wheat H3 gene (pTH012) ([3,4] and see table 1). Moreover, in the H3 gene this sequence (−134 to −89) has been shown to function as a *cis* element (Nakayama, T. et al., unpublished). This suggests that HBP-2 may be a *trans*-acting factor(s) for the transcription of both the H3 and H4 genes.

To determine whether this is so, we performed cross-competition experiments with the mobility shift assay (fig.3). When an unlabeled H4 (−108 to +51) or H3 (−167 to −41) fragment that contains *cis* elements was used as the competitor,

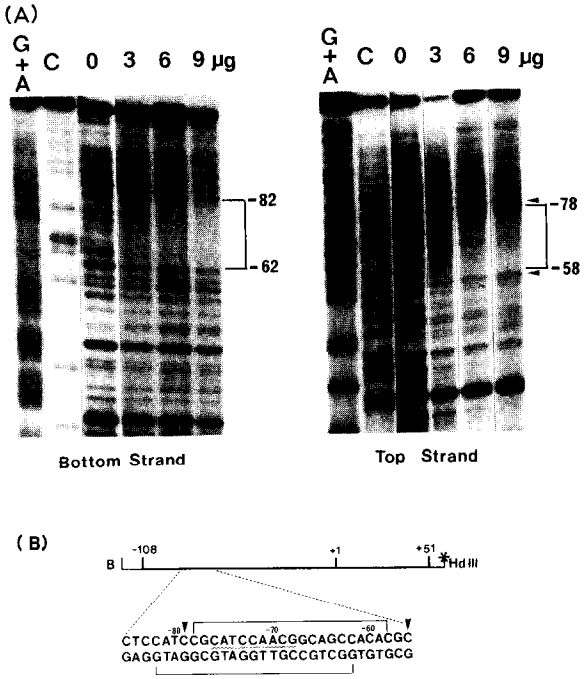

Fig.2. DNase I footprint analysis of HBP-2 binding to the 5′-flanking sequence of the H4 gene (pTH011). (A) DNase I cleavage patterns of the H4 probe (−108 to +51). Lanes G+A and C, chemical cleavage ladders. Other lanes, DNase I cleavage in the absence, or presence (3–9 μg protein/assay), of the 0.35 M KCl heparin-Sepharose fraction. DNase I-protected regions are shown by the vertical lines on the right. Arrowheads indicate the DNase I-hypersensitive sites. (B) Sequences in the HBP-2 binding region estimated from DNase I footprinting of the H4 gene. Protected regions are shown by the horizontal lines alongside both strands. DNase I-hypersensitive sites are shown by the arrowheads. The wavy line indicates the putative nonameric sequence (see text).

HBP-2 binding to H4 probe was prevented (fig.3, lanes 3,4); however, binding was not prevented when the oligonucleotides that contain the hexameric or octameric motif, or a pUC118 polylinker fragment were used (fig.3, lanes 5–7). This indicates that HBP-2 is a sequence-specific DNA-binding protein(s) which recognizes the common motif in the 5′-flanking regions of the H3 and H4 genes and that it is distinct from HBP-1, a hexamer-specific DNA-binding protein(s) [7]. Our unpublished data on DNase I footprinting of the H3 fragment show that HBP-2 can bind to the *cis* element of the H3 gene described above.

In the research reported here, we did not perform experiments to detect the HBP-2 contact sites

Table 1

Comparison of nonameric homologies in several plant genes including histone genes

Plants	Genes	Sequences	References
Wheat	H3 (pTH012)	−183 −166 CCATCCGCATCCAACGGC	[4]
	H4 (pTH011)	−150 −134 CCATCCGCATCCAACGGC	[3]
Corn	H3 (C4)	−197 −177 TCCATCTCCATCCAACACCTA	[8]
	H4 (C14)	−193 −181, −146 −135 TCCCATCCAACGGT, CCCCATCAACGC	[9]
	H4 (C7)	−165 −153 GCCCATCGAACGGC	[9]
Arabidopsis	H3 (A713)	−152 −142 AACATATAACGA	[10]
T-DNA	octopine synthetase	−167 −157 ACATCCAACGT	[11]
Potato	sporamin (gSPO-1)	−249 −238 TCCATCCATCGG	[a]
Soy bean	β-conglycinin α′-subunit (Gmg17.1) β-tubulin (Sβ-1)	−196 −183 ACATTCACCAACTC −251 −237 CAATCATGCAACTTC	[12] [13]
Consensus		5′ CCCATCCAACGG 3′	

[a] Hattori, T. et al., personal communication

Nucleotide sequences are written in the 5′- to 3′-direction, with respect to the top strand. The positions around the nonameric sequence are negatively numbered from the first nucleotide of a translation initiation codon; homologous sequences are underlined

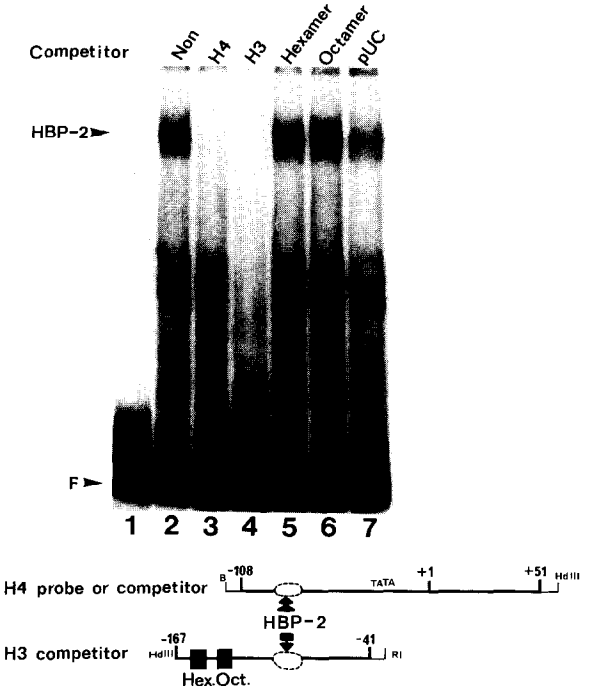

Fig.3. Competition experiments on HBP-2 binding to the H4 upstream region performed with the mobility shift assay. Lanes: 1, free probe; 2, binding assay in the absence of competitor DNA; 3–7, binding assays in the presence of competitor DNA: a 25-fold molar excess of the specific H4 (lane 3) or H3 (lane 4) competitor, a 50-fold molar excess of the non-specific oligonucleotide competitor containing the hexameric (lane 5) or octameric (lane 6) motif, and a 50-fold molar excess of non-specific pUC118 polylinker competitor (lane 7). The binding reaction contained 0.35 M KCl heparin-Sepharose fraction (3 μg protein/assay). Fragments used as the probe and as competitors are shown at the bottom. F, free probe; HBP-2, specific protein(s)-DNA complex.

in the H3 and H4 genes, or to determine the biological functions of HBP-2. We did find that the 5′-upstream regions of certain other plant genes have the sequences that are highly homologous to a characteristic nonameric motif present in the HBP-2-binding region (table 1). In the β-conglycinin α′-subunit gene, for instance, the nonameric motif is part of the *cis* element [12].

We therefore propose that the nonameric sequence is a *cis*-acting regulatory element for the transcription of some plant genes.

Even if HBP-2 is demonstrated to be a *trans*-acting factor(s), it may not necessarily be specific to plant histone genes because nonameric sequences have been found in other plant genes. HBP-2 may function in cooperation with HBP-1 in the transcription of plant histone genes as both DNA-binding proteins interact with the *cis* elements of histone genes. We are now engaged in further purification of HBP-2 in order to determine its biochemical characteristics.

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